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Analyzing surface protein expression and internalization

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ENTITLED

Analyzing Surface Protein Expression & Internalization

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF

BACHELOR OF SCIENCE IN BIOENGINEERING

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ABSTRACT

G-protein coupled receptors (GPCRs) are currently the largest class of membrane receptors and are targeted by a majority of the modern drug therapeutics. In addition, they partake in many physiological and pathological processes such as inflammation, growth, and hormone responses. Most importantly, GPCRs are targets of many disease-specific pathways such as Alzheimers, hypertension, leukemia, and depression. As a result, there is an immense interest in studying GPCRs as this area provides further knowledge into the specific disease pathways and allows the discovery of novel therapeutics. In order to have a better understanding of pathways, scientists have studied GPCR activation. The prostanoid receptors are of great interest because they coordinate a vast range of physiological processes such as regulating cardiovascular pathways, modulating neuronal activity and controlling immune responses. We collaborated with Multispan Inc., a biotech company focusing exclusively on drug discovery research targeting GPCRs, to monitor the agonist-induced internalization of GPCRs from the prostanoid family through live cell flow cytometry. From our experimental results, we have observed over 50 percent internalization for the EP1 and EP4 receptors within 60 minutes after being activated by iloprost and PGE_2 respectively. Our initial experimental results have also shown over 40 percent internalization for the TP receptor within 120 minutes after exposure to PGD_2. Overall, we have been able to utilize Multispan's proprietary cell lines to overexpress a few of the prostanoid receptors. These assays are powerful tools for the discovery of novel therapeutics, as they enable the testing against libraries and screening from a few thousand to a few million compounds.

Keywords: GPCR, prostanoid, agonist, internalization, flow cytometry, iloprost, PGE_2

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We would also like to thank Dr. Zhang for connecting us with this unique opportunity to collaborate with Multispan Inc. for our senior design project.

We would like to thank the School of Engineering and the Bioengineering Department for their ongoing support throughout our college careers. This project is funded in part by the School of Engineering.

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1.0 INTRODUCTION

1.1 Background/Motivation

G-protein coupled receptors (GPCRs) are the largest and most diverse class of membrane receptors. One of the main functions of GPCRs is to trigger secondary messengers such as cyclic adenosine monophosphate (cAMP) or calcium ion channels. The activation of the secondary messengers helps regulate body functions such as sensation, growth and hormone responses.[1] In order to trigger secondary messengers, the GPCR must be activated by the binding to a ligand. As a result, there is an immense interest in studying the specific pathways of GPCRs. GPCRs also have an extremely large market of about $122 billion by 2018. Currently, over 250 GPCRs have been identified and 150 GPCRs to de-orphanize, which presents a tremendous opportunity for future research.[2] The biggest motivation for GPCR research is that they are responsible for many disease-specific pathways such as endocrine and vision disorders.[3] As a result, the industry has a demand for better therapeutic discovery tools, such as assays, as a new platform for monitoring receptor activation. These new platforms would lead to a better understanding of the disease pathways. By developing a better understanding of disease pathways, researchers will be able discover novel therapeutics for the treatment of these diseases. Please refer to the glossary of terms in

1.2 Project Goals & Objectives

Our aim is to develop quantitative measurements of receptor response for novel therapeutics. This is done first through the development of assays to measure the internalization of GPCRs. The process of internalization begins when the GPCR is activated through the binding of a specific ligand at the site by the N-terminus. By measuring the EC50 values for internalization for each specific GPCR, we can better understand an entirely new platform of receptor activation. In addition to EC50 values, we must obtain the specific time intervals at which internalization occurs. By obtaining these key pieces of information, we hope to establish new internalization assays using Multispan Inc.'s existing platforms. As

interns at Multispan Inc., we are responsible for developing assays for the GPCRs within the prostanoid family. Our aim is to obtain valid EC50 values for the activation of these two families of GPCRs. EC50 values must be reproducible within three folds of each other. If the previous goals are achieved, our future goals would be to perform antagonist testing to inhibit the internalization of the GPCRs. If the previous two tasks are accomplished we can then use this platform to test against pharmacological libraries for novel therapeutics.

1.3 Review of Field/Literature

The following paragraphs contain our literature findings on the general structure of GPCRs and the specific functions and diseases associated with prostanoid receptors. In addition, we researched on the ligands that have been used for specific GPCRs for other assays such as calcium and cAMP assays. These findings helped us in our agonist selection along with targeting the time frame for running these experiments. Most importantly, our literature research helped us select the GPCRs with the highest chance to internalize with the available agonists that we have.

1.3.1 General Structures of GPCRs

GPCRS are composed of seven hydrophobic transmembrane domains which include an extracellular amino terminus and an intracellular carboxyl terminus. A ligand binding domain, located close to the extracellular amino terminus, causes the activation the GPCR when a ligand binds to the region.[4]

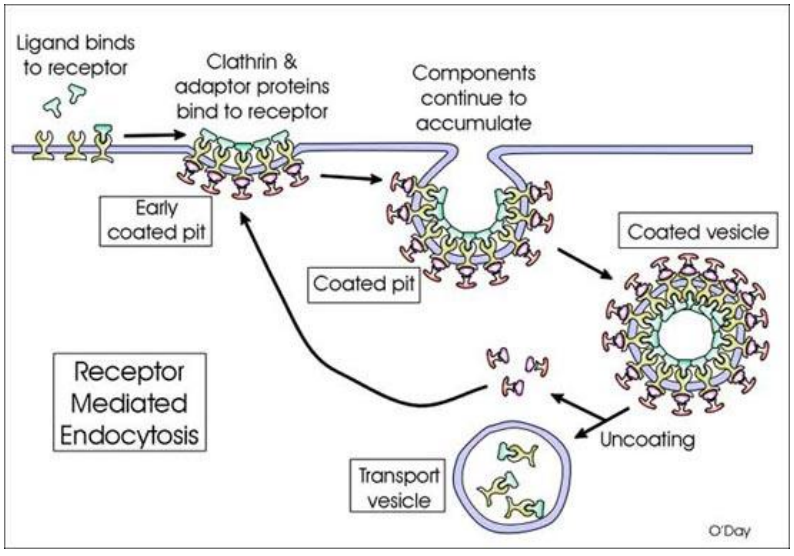

Figure 1: Schematic showing the different steps in clathrin-coated endocytosis

The process of internalization occurs when a ligand binds to the GPCR. Clathrin-dependent GPCR internalization occurs when an agonist binds to the receptor and triggers conformational change because it transforms the receptors into substrates of the GRKs. The GPCRs that are occupied by the ligand become phosphorylated and at the cytosolic Serine and Threonine residues. The phosphorylated ligand-complex then rapidly recruits β-arrestins which disrupts further signaling to the G proteins. β-arrestins then promote clathrin-dependent endocytosis by binding to the GPCR with adaptor proteins to form the early-coated pit. As illustrated by Figure 1, more clathrin and adaptor proteins continue to accumulate and the clathrin-coated pit detaches from the plasma membrane with the addition of dynamin. The ligand-receptor clathrin complex then internalizes and travels along the internalization pathway. The clathrin and adaptor proteins then uncoat from the complex and from this point, at least two possibilities exist; the receptors are either removed from ligands and recycled back to the cell surface or the receptors are transferred to late endosomes which readied them for lysosomal degradation.[5]

1.3.2 Prostanoid Receptors

Prostanoid receptors are part of a family of active lipids which can be further divided into three main groups: prostaglandins, prostacyclins, and thromboxanes. They are sometimes referred to as local hormones which act in an autocrine manner to control the effects of other hormones in the circulation. They coordinate a vast range of physiological processes which ranging from producing cardiovascular effect to modulating neuronal activity, and controlling inflammation and immune responses. The receptors express their activity by interacting with G-protein-linked receptors divided into five classes. PGE_2(EP1 through EP4), PGD_2 (DP1 and DP2), $PGF_{2\alpha}$ (FP), PGI_2 (IP) and TXA_2 (TP). The initial effect in activating these receptors is the increase or decrease in the uptake rate of cAMP or calcium ions. The activation of the receptor further translates into regulating the physiological and pathological processes of the body.[6] The disease pathways associated with prostanoids include: atherothrombosis, aortic aneurysm, cancer,

hypertension, and leukemia. An example of how prostanoid receptors may play a direct role in cancer is displayed in mice model. Mice models lacking EP_1, EP_2, or EP_4 have a lower chance of disease in carcinogenesis models.[7]

1.3.3 Selection of agonists for prostanoid receptors

Our approach towards agonist selection would be to research on other assays that have been done for the specific receptor. Prostaglandin E_2 (PGE_2) has been found to be an effective agonist to trigger internalization for the EP4 receptor. In fact, it has been observed that there is about 20 percent internalization for HEK 293-EBNA-HA-hEP4 transfected with dominant negative β-arrestin 1. In addition, 1μM of PGE_2 was used over the course of 60 minutes.[8] Although the specific cell line is different along with the transfected components, these are great starting points to construct our internalization assay for EP4 receptor. EP1 and IP receptors have been found to have an extremely high binding affinity for iloprost. High binding affinity may potentially play a large role in internalization as the experiments within an article by Whittle et al., has tested the EC_{50} values for cAMP and calcium assays. On the other hand, iloprost has a low affinity for FP, EP_3, and EP_4 receptors.[9] PGD_2 has been found to be an effective agonist for DP_1 and DP_2.[10] In addition, the potency of the agonists found above has been validated through the IUPHAR database.

2.0. SUBSYSTEM CHAPTERS

2.1. Technology Overview

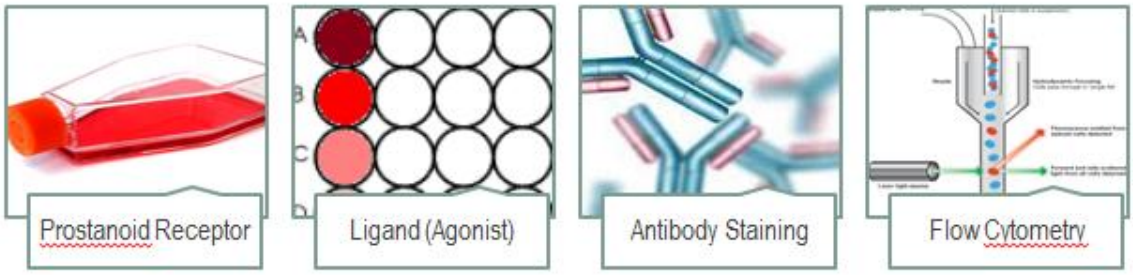

Figure 2. A linear flow diagram illustrating the technology and processes involved in the entire experiment. First, the receptor of interest is chosen and then the ligand potent to the receptor is incubated with the cells overexpressing the desired receptor for a given amount of time. Next, subsequent washes are performed and the cells are stained with anti-FLAG tag antibodies. After antibody staining, the cells are ready for analysis under the flow cytometer.

Our experiment is composed of several components: receptor and ligand selection, incubation, subsequent washes, antibody staining, and analysis of under the flow cytometer. Serial dilutions are performed to prepare the drug compounds and a detailed description can be found in chapter 3.1. The drug compounds are then incubated in 37 degrees Celsius with the cells overexpressing the receptor of interest and then subsequent washes are performed. The cells are then stained with the anti-FLAG tag antibody and incubated for 30 to 60 minutes. More subsequent washes are performed and the cells are resuspended in FACS buffer and ready for analysis under the flow cytometer. The data obtained is then analyzed in Graph Prism 4 to create the EC_{50} plots.

2.3 Customer Needs and Requirements

2.3.1. Company Needs

When we first started the assay development, we realized that Multispan Inc., was the customer who we need to prioritize first because we knew very little about the technology and the product market. As a result, we interviewed three

different individuals within the company and created three different questionnaires which can be found in *Appendix A.* Instead of sending out e-mails, we interviewed each one individually so that we immediately clarify our questions if they are unclear. They individuals from the company range from different areas of specialties such as research and development, management and sales.

We first interviewed our industry advisor and Multispan Inc's CEO, Dr. Helena Mancebo. Her feedback would lead us to our main task and what is expected from our team. In addition, we would find out what we need to do to address the needs of Multispan Inc's customers. Dr. Helena Mancebo provided us with the following information:

Currently, there is not a direct competitor offering the same internalization assays as Multispan Inc. The results of each internalization assay must be reproducible with less than 3 fold differences for EC50s for IC50s in 3 independent experiments for it to be considered "validated." There is a need to develop validated internalization assays for all targets within each assigned receptor family.

The second individual who is interviewed was Dr. Ricai Han, who was the Senior Scientist at Multispan Inc. Although Dr. Han has not directly worked with Prostanoid receptors, he was able to provide us with some of his insights from a researcher's perspective. According to Dr. Han, Multispan Inc, is unaware of the affinity of the receptor or any other specific information regarding the drugs sent by companies such as Johnson and Johnson for testing. They also do not know which compound could trigger signal transduction from the GPCR. In order to perform internalization assays on these drugs the researcher must characterize the function and the activities of how it could affect surface expression in addition to finding the internalization factor. Without performing the previous steps, it

would be extremely difficult to select for the most suitable GPCR that the drug acts upon.

Finally, we interviewed Dr. Radhika Venkhat, who is the R & D Manager at Multispan Inc. Dr. Venkhat has provided us with an entirely different perspective as she is the expert in internalization assay at the company. According to Radhika, she has worked with internalization many different types of receptor and the approach has to be paired with understanding the different properties of each specific receptor. She has only worked with DP2 from the family that we are assigned to work on. She explains to us that one of the first issues we will face is the selection of the specific agonist or antagonists to use for each assay. These are chosen based on those reported from literature research and analysis. Some may require internalization with other receptors or it is derived from external factors. The second issue is the framing the time point of internalization for an unknown compound with a receptor. The main reason is that for some receptors, there is a rapid recycling time frame for some receptors and it is essential to grasp the window of internalization in order for us to not miss it. She advised our group to keep practicing on the technique and running the assays as once the technique is mastered and we learn more about the theoretical component from the literature, we will have a much better understanding on how to set up the assays.

2.3.2. Product Design Specification

Our Product Design Specification is broken into 3 categories: the assay itself, confidentiality, and deliverables. A detailed table is provided in *Appendix A.* The assay itself further contains 3 subclasses: EC_{50} or IC_{50} (optional) accuracy, complete validation of all family receptors, and reproducibility. Confidentiality is further divided into 3 subclasses: proprietary procedures, materials and information, and IP protection of ligand specifics from pharmaceutical companies. The last category is deliverables which includes one subcategory of having complete data under final conditions.

2.4 Benchmarking Results

Currently, there are not any known existing products that are similar to the internalization assays offered by Multispan. The most significant advantage of live cell flow cytometry is that it allows the accuracy of the quantitative analysis to go down to counting each individual cell over a specific time period. Currently, there isn't another testing method that is able to provide such accuracy. Although this testing method is lengthier compared to the alternatives, the optimum accuracy obtained from the measurements makes this a worthwhile tradeoff. Other GPCR companies perform assays on different GPCR lines which are not screened by Multispan Inc. The testing methods used are also different as none of them currently incorporate the same platform with live cell flow cytometry and fluorescence tagging as Multispan Inc. Another indirect competitor would be virtual drug screening which is extremely promising in that it reduces the costs and time for novel therapeutic screening but it requires the assays to be done and validated previously. Table 1 below compares the advantages and disadvantages of the testing method that Multispan Inc. uses to the other available testing methods.

Table 1. Analysis of different testing methods: Table comparing both the advantages and the disadvantages between the other available testing methods and Multispan Inc.'s testing platform

Testing Method	Advantages	Disadvantages
Multispan's platform Live cell flow cytometry	Flow cytometry enables precision down to each individual cell for monitoring internalization and provides specific time points for internalization	Assay is more time consuming for some receptors
Other platforms	Often little complex analysis	Quantitative results

(Infrared fluorescence)	involved and direct functional read out.	are not as precise down to individual cell count.
Virtual Drug Screening	Can cut costs and time and is and provide a theoretical result	This requires a valid existing database to build on and is not effective yet as a predictor tool.

2.5 Key System Level Issues

2.5.1. Main Issues:

The main issue of the internalization assay system revolves around the uncertainty of how the ligand will internalize. Although the selection of the ligand is made based upon known publications, the way of which these receptors internalized is still unknown. The success of using a particular ligand for different assay does not imply that the similar success will carry over for internalization. In addition, it is more economical to use the ligands that Multispan Inc. already has rather than buying more since it's not guaranteed that those will work really well. To further complicate matters, some G protein-coupled receptors require homodimerization or heterodimerization with another receptor in order to internalize into the cellular membrane. After figuring out the specific mechanism and conditions which triggers a ligand to internalize, the next issue would be to pinpoint the time frame at which this event occurs. Some receptors may internalize within 5 minutes while others may require a much lengthier period such as 3 hours in order for the process to occur. Although an EC_{50} curve is able to guide us into the right time point, knowing the exact window would cut down costs and time for the experiments.

2.5.2. System Options:

Agonist selection: The selection of the agonist is done through researching similar experiments which have been performed on the specific prostanoid

receptor. We will work on the receptors with known agonists for similar experiments first before we move onto the other ones.

Concentration: The concentration of the agonist compound can be varied accordingly. A higher starting compound concentration would be used if the agonist has a low affinity towards the receptor. If the agonist has a high affinity towards the receptor, a low starting concentration would be required.

Specific Time period: The time period of internalization is obtained through literature research which contains time intervals for other assays such as calcium, and cAMP. For receptors that contain little information a time window of 2 hours is general accepted.

2.5.3. Tradeoffs:

Currently, the experiment contains several paramaters which need to be taken in consideration as the assays may completely head towards a different direction with the inclusion or inclusion of these factors. Table 2 highlights the pros and the cons of three options such as cell fixation, flow cytometery, and an economical agonist selection.

Table 2. Tradeoffs: Comparison between each process option and the decision made in the end with both the pros and cons in consideration

Option	Pros	Cons	Decision
Fixing the cells with paraformaldehyde	Allows assays to be done at later time	Too many cells are lost in the process and you need to monitor the timeframe carefully	Fixing will not be considered for experiment
Live Cell Flow Cytometry	Providing analysis down to each individual cell	Much more time consuming than using a plate reader	Flow cytometry will be used because of accuracy

Using alternative agonists over the most potent ones found in literature	More economical to use what the company has first	Assays may not work well with alternatives because they are not as potent with the receptor	Alternative agonists will be used first before we venture into buying the most potent ones found in literature

2.6 Team and Project Management

2.6.1. *Project Challenges and Constraints*

In any group project, challenges and constraints are inevitable but they must be taken in consideration and solved before the project can progress. The major constraint for most projects is the management of the given budget. Fortunately, most of the expensive compounds, cell lines, and reagents were provided by Multispan Inc. By communicating with our industry advisor Helena Mancebo, on a regular basis, we were able to avoid this issue for our project. The main challenge that we faced is time constraint. As seniors, we are faced with the challenge of managing our time efficiently between working on our senior design project, finding and securing jobs, completing graduate school applications, and working on homework and group projects for classes. As a result, it was extremely difficult on occasions for us to do our best work to meet the deadlines for specific portions of our senior design project. To address this issue, we scheduled regular group meetings and divided up the portions of the project to get the assignments completed and turned in on time. It was difficult to meet up as a group during the winter quarter because our class schedules were different and we were extremely occupied with interviews and outside group projects. As a result, we did not work together a lot for the project during the winter quarter because the experiments required a large time slot and we did not have a timeslot where we were both free. To remedy this issue, we updated each other

regularly on the results and alternated with each other in changing the media and trypsinizing our cell lines.

The second challenge that we faced is travel to and from the company. It became extremely difficult for us to adjust to the long drive to and from Multispan Inc., which is headquartered in Hayward, California. We must schedule our classes wisely so that we have an ample amount of time to work on our assays and to avoid the traffic hours when we are driving down to the company site. As a result, we struggled immensely during the first quarter because of the limited amount of time that we had to drive up to the company. Molecular biology requires a lot of practice on the technique and we didn't spend enough time the first quarter. As a result, our main challenge for the most part is the limited amount of time to research, develop adept lab skills, and to carry out experiments. Although we had some cell culture experience prior to our project at Multispan Inc., we never maintained multiple cell lines over months. The added challenge is to maintain multiple cell lines 40 minutes away from where we live. Contamination or unhealthy cells would cost both time and money to replace.

2.6.2. Team Budget

Because we are collaborating with Multispan Inc., on this project, it is fully funded by the company because they provide costly cell lines, reagents and chemicals for our project. For example, we shouldn't take more than we actually need of chemicals and reagents such as buffer, antibody, and drug compounds because those are extremely expensive to waste. This would also push back the project if we run out of chemicals and reagents. We also need to take an economical approach when we run the experiments and use what we have first. It is not logical to purchase the most potent agonist for a GPCR without using the alternative agonists in the inventory because it's guaranteed that the assay will work extremely well with the agonist purchased. A detailed breakdown of the budget can be found in *Appendix*

2.6.3 Timeline

It is extremely important to construct and follow a project timeline to ensure that the team is staying on top of things. However, it is often rare that the project timeline is followed through in every step so the timeline has to be adjusted accordingly. The main issue we faced for the timeline is on figuring out exactly how much time is required for each specific task. As a result, we decided to create an outline by a week-to-week basis which works a bit better for our project. For the most part we were on task with the outline for the week-to-week tasks but we fell short sometimes because we underestimated the amount of time required to complete the task. A detailed outline can be found in *Appendix B.*

3.0. SYSTEMS INTEGRATION & VERIFICATION

3.1. Experimental Protocol

3.1.1. Expression of Prostanoid Receptors

Mammalian cells overexpressing the receptor of interest were acquired from Multispan's proprietary cell lines. Human embryonic kidney cells from the HEK 293T cell line have been modified to overexpress the receptor of interest. Figure 2 illustrates a general structure of this specialized cell line. The process of receptor overexpression is achieved by inserting a plasmid containing the complementary DNA (cDNA) sequence coding for the receptor with the addition of 24 base pair coding for the octapeptide FLAG-tag at the N-terminus. Plasmid also included selection markers puromycin and ampicillin resistance to ensure the exclusive survival of cells that have taken up the plasmid. The translation of this plasmid was ensured by the presence of a strong promoter.

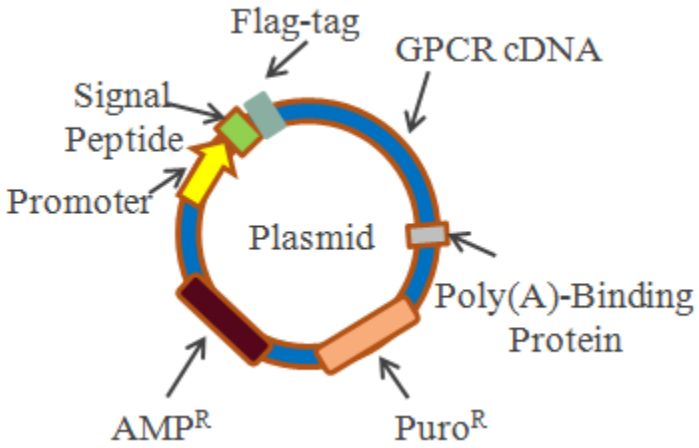

Figure 3. Plasmid used to overexpress receptor of interest. This process was performed at Multispan Inc.

3.1.2. Activation of Prostanoid Receptors

There are three types of receptors in the prostanoid family, inhibitors of adenalyse cyclase, contractile and relaxant receptors, with total of eight different receptors identified. We have focused on the activation and the subsequent internalization of Prostaglandin E Receptor 4 (EP4), Prostaglandin E Receptor 1 (EP1) and Thromboxane Receptor (TP). Utilizing available literature we have identified compounds that activate each of these receptors, known agonists, as well as reported EC50 values. We created dilutions of the compounds to encompass a wide enough concentration window to achieve acceptable EC50

values; in this case a 1 in 5 dilution was appropriate. The EP4, EP1 and TP receptors were activated with Prostaglandin E2 (PGE2), Iloprost, and Prostaglandin D2 (PGD2), respectively. Table 1 below displays the different starting concentrations and dilution rates were used for each compound to create 5x concentrations.

Table 3. Dilutions of the different compounds used for EP1, EP4, and TP. The concentrations are in micromolarity with the highest concentration being five times as concentrated so that the last dilution step causes the final concentration to be 1x.

Receptor	Agonist	Dilution Rate	Highest 5x Concentration (μM)	Lowest [5x] (μM)	Highest [1x] (μM)	Lowest [1x] (μM)
EP1	Iloprost	1:5	5	$3.2\ e^{-4}$	1	$6.5\ e^{-5}$
EP4	PGE_2	1:5	2.5	$1.6\ e^{-4}$	0.5	$3.2\ e^{-5}$
TP	PGD_2	1:5	1000	$6.4e^{-2}$	200	$1.26e^{-2}$

In order to create the appropriate concentrations two steps dilution were made. Research compounds are often dissolved in esters. To ensure the solubility of the compounds, two steps dilutions were made first into dimethyl sulfoxide (DMSO) followed by phosphate buffer saline (PBS). Precautions were taken to ensure the final concentration of DMSO in the media during incubation periods did not surpass 1% DMSO. The only exception to that is for iloprost because the stock compound came in methyl acetate, an alcohol, so 3.5% DMSO is needed. To control for the addition of DMSO to the cells the eighth and final well did not contain agonist compound and was comprised of DMSO and PBS. This can be compared to the seventh well to ensure the values are close.

During the incubation period cells overexpressing the receptors were incubated with 40ul of Dulbecco's Modification of Eagle’s Media (DMEM) with 10% Fetal Bovine Serum (FBS). Approximately 30,000 cells were placed in each well of a

96-well plate.10ul of the 5x dilutions, for a total of 50ul, were then added at 30 minute intervals ranging from 30 minutes to 120 minutes. The media was then removed and washed with PBS three times, ensuring that everything is done on ice, before proceeding with staining.

3.1.3. Tracking of Prostanoid Receptors

3.1.3.1. Antibody Staining

Due to the modification of the GPCR done my Multispan we can track its expression on the surface using the Anti-FLAG-tag Antibody that bind to the available Flag-tag. Anti-FLAG-tag Antibody (Prozyme α Flag 1.13mg/ml) was acquired in 500x concentration. Anti-FLAG-tag Antibody was diluted in PBS to 1x concentration and 150ul was added to each well. Cells were placed in a dark refrigerator at 2 degrees Celsius for 30 minutes to allow antibodies to bind. Two washes were then performed to remove Anti-FLAG-tag Antibodies with PBS and one was with PBS + 1% Bovine Serum Albumin (BSA) to prepare samples for FACS (Fluorescence-activated cell sorting) analysis.

3.1.3.2. Analysis of Internalization

Live cell flow cytometry was performed on the samples to analyze the bind of Anti-FLAG-tag Antibodies to the available Flag-tags on the receptors present on the surface of the cell. Receptors that have been activated and internalized will correlate to a decrease in the mean fluorescence intensity (MFI). Every cell passes an interrogation point in which the size, complexity and fluorescence are analyzed, we counted 2,000 of these events and obtained a MFI value for population comparison.

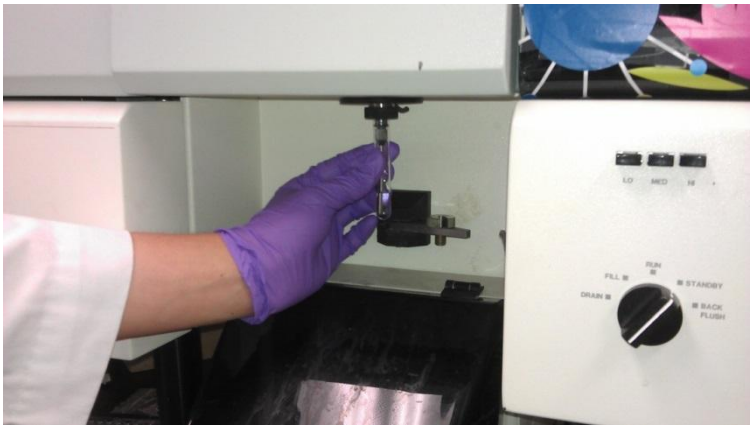

Figure 4. Loading of the cells overexpressing the GPCR of interest on the BD scientific FACsort

Figure 4 illustrates the loading of a sample containing cells overexpressing a GPCR receptor and pre-stained with antibody. The instrument used was a BD Scientific FACSort and the analysis was run on BD CellQuest Pro software. Parent cells in which the overexpressed receptor was induced were first analyzed to provide background fluorescence

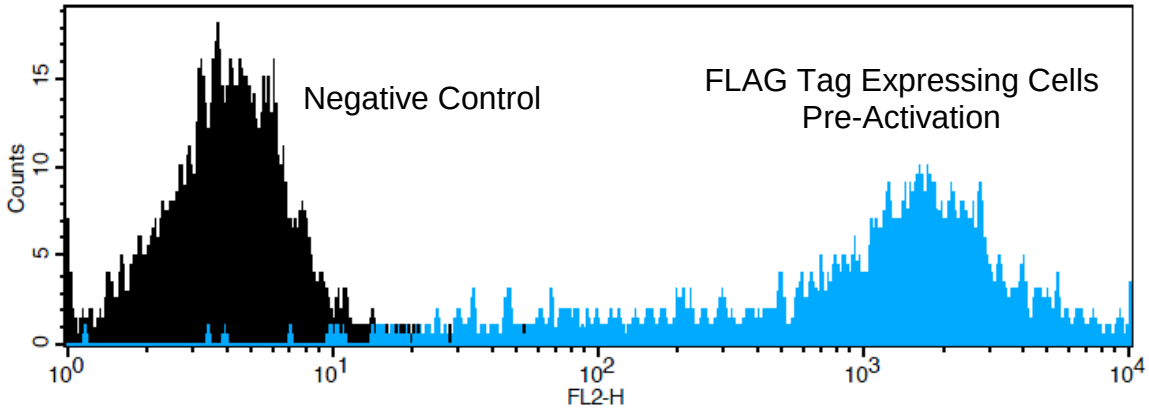

Figure 5. Histogram showing the the MFI of the parental cells, representing our negative control (black), and histogram FLAG tag expressing cells prior to the addition of any drug compounds, taken as positive control for the experiment (blue).

reading and calibration. The fluorescence in these cells is very low. We can then analyze the fluorescence of cells with the overexpressed receptor-Flag-tag complex. If the cells are correctly expressing this complex then the Anti-Flag-tag Antibody will be able to bind and cause an increase in fluorescence.

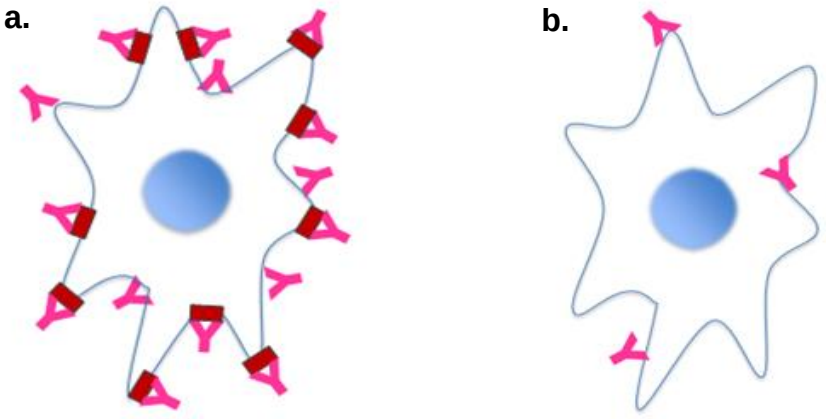

Figure 6. Side by side comparison showing how the Anti-FLAG tag antibody works on (a) FLAG tag GPCRs and (b) parental cells not expressing GPCRS

Figure 5 above shows a histogram which includes the MFI for the parental cells in black and the Flag tag expressing cells pre-activation. The MFI for the parental cells is close to zero because the cells do not overexpress any GPCRs. As a result, there is a need to subtract the addition of the parental cell MFI when calculating the percentage of internalization. Figure 6a. above illustrates how the Anti-flag tags bind to the Flag-tag GPCRs which increase the fluorescence intensity. Figure 6b. illustrates how parental cells on attracts little or none of the Anti-flag tags which is why the fluorescence intensity should be close to zero. A few reasons why the MFI is not zero for the parental cells is due to the error in the instrument and a few antibodies clinging to the parental cells.

3.2. Results

A BD scientific FACSort was used to analyze the mean fluorescence intensity (MFI) of our experiments. The data is then plotted on Graph Prism version 4 and fitted with a sigmoidal dose response varying slope. Log of the different concentrations of the compound is plotted against MFI and In the first experiment, we worked with EP4 receptor. We incubated the receptor with prostaglandin E2 (PGE_2) for 30 and 60 minutes. As observed in Figure 7b. over 50% internalization is observed during 30 minutes with an EC_{50} value of 5.21×10^{-9} M. Triplicate experiment was performed with the error bars representative the deviation between each experiment's concentrations. Figure 7b represents the MFI converted to percentage of internalization by first subtracting each value by the parental MFI and dividing by the MFI of each concentration by the negative control. Figure 7a. and 7c. represents the MFI before the exclusion of the parental MFI for each respective time interval. In Figure 7d. over 50% internalization is also observed at 60 minutes with an EC_{50} value of 5.527×10^{-9} M. Because the EC_{50} values of both time points are extremely close to one another, we cannot exclude either. The complete plot displaying the all the dose response curves can be found in *Appendix E.*

3.2.1. EP4 Internalization

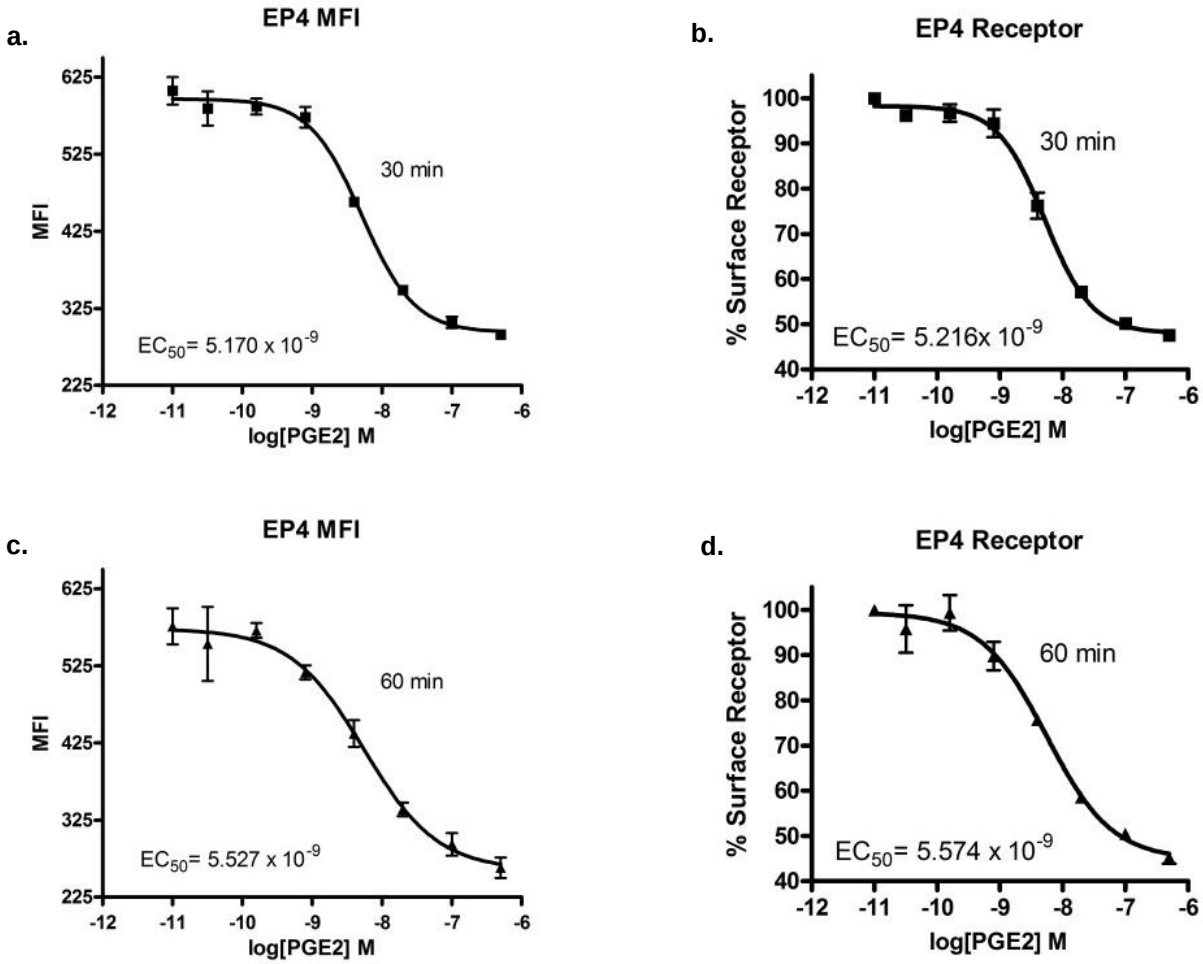

Figure 7. EC_{50} plots of EP4 internalization at 30 and 60 minutes. (a) & (c)) Log of the concentration of PGE_2 in molarity is plotted against MFI before the exclusion of parental MFI for both 30 and 60 minutes. (b) & (d) represents the log of concentration of PGE_2 plotted against percentage of internalization. Experiments are done in triplicates.

3.2.2. EP1 Internalization

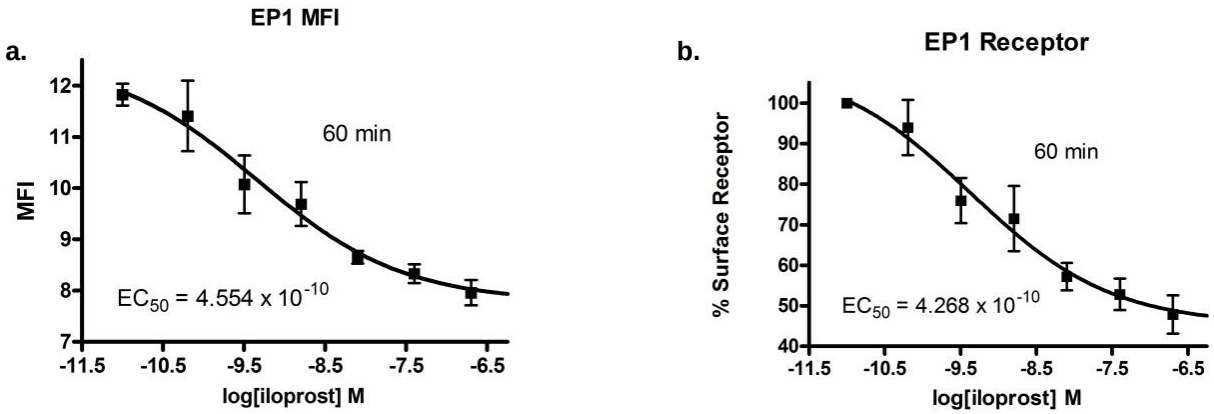

Figure 8. EC_{50} plots of EP1 receptor internalization after 60 minutes. (a) Log of concentration of iloprost in molarity plotted against MFI. (b) represents the log of concentration of iloprost plotted against percentage of internalization. Experiments are done in triplicates with error bars representing deviation from each (a) MFI and (b) percentage of internalization.

In the second set of experiments, we incubated EP1 receptor with iloprost for 60 minutes. As observed in Figure 8b. over 50% internalization occurred with an EC_{50} value of 4.27×10^{-10} M. Figure 8a. represents the EC_{50} value obtained for MFI value without the exclusion of the parental cell MFI. The preliminary run which includes 30 minutes can be found in *Appendix E.*

3.2.3. TP Internalization

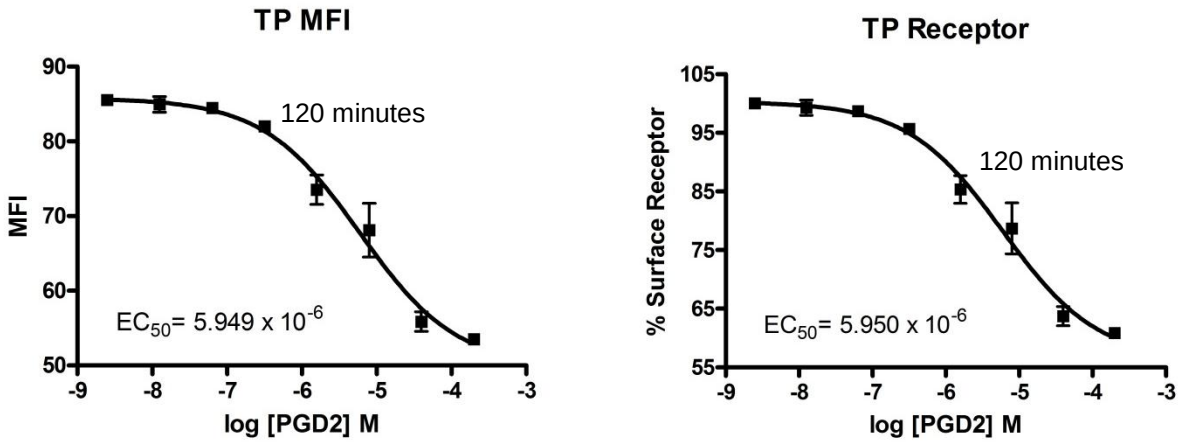

Figure 9. EC_{50} plots of TP internalization after 120 minutes. (a) Log of concentration of iloprost in molarity plotted against MFI. (b) represents the log of concentration of iloprost plotted against percentage of internalization. Experiments are done in duplicates with the error bars representing deviation from each (a) MFI and (b) percentage of internalization

In our last successful experiment, TP receptor was incubated for 120 minutes with prostaglandin D_2 (PGD_2). As observed in Figure 9b. over 40% internalization occurred with an EC_{50} value of 5.95×10^{-6} M. Figure 9a. represents the EC_{50} value obtained for MFI value without the exclusion of the parental cell MFI. The preliminary run which includes 30, 60, and 90 minutes can be found in *Appendix E.*

3.2.4. Unsuccessful Experiments

Besides the EP1, EP4, and TP1 receptor, we also ran internalization assays for EP2, DP1 receptor but little internalization was observed or the experiment was not repeatable. The data for these experiments can be found in *Appendix E.*

3.3. Analysis

3.3.1. EP4 Analysis

With over 50% internalization in 3 separate sets of experiments, the results appear to be extremely promising in that the EC_{50} values fall within 3 fold of each other. Our starting point for concentration was based on the International Union of Basic and Clinical Pharmacology's (IUPHAR) database which listed PGE_2 as having a binding affinity of 9.0 for EP4 for other assays. Internalization assays tend to have a higher number and this binding affinity value is converted to the negative power of base 10 to derive the exact concentration. As a result, an EC_{50} of around 5.17 and 5.53 nM is reasonable. Because the deviation between the MFI of each dilution is extremely small, we can conclude that internalization does indeed happen within this time window and concentration. Neither 30 nor 60 minutes can be omitted because of the close proximity between the two EC_{50} values.

3.3.2. EP1 Analysis

Over 50% internalization is observed in 3 separate sets of experiments and the EC_{50} values of each individual experiment fall within 3 fold of each other. In addition, our starting point for this assay was based on both IUPHAR and Multispan Inc.'s value for other assays such as calcium and cAMP. Our EC_{50} of 4.27×10^{-10} M value even falls within 3 folds of Multispan Inc.'s EC_{50} value. None of the error bars deviate too much so the assay is validated for this receptor with iloprost as the agonist.

3.3.3. TP Analysis

We cannot entirely state that this assay has been validated because we were only able to reproduce the results in 2 sets of experiments. The deviation in error is extremely small which makes this assay promising. The different results obtained from the third experiment may be due to pipetting error or slight contamination in between. Repeating this experiment will be one of the future goals for the group that takes over this project.

4.0 ENGINEERING STANDARDS AND CONSTRAINTS

4.0.1. Social

It is our duty to ensure that our internalization assays are reproducible with less than a 3 fold difference for the drug receptors IC_{50} and EC_{50} values. In addition, our assay has to be robust with time intervals easily adjustable for testing on other drug compounds. Our project does not have any end users outside of our testing facilities. We must ensure that others inside of the company can reproduce our assays and utilize them for drug discovery much after our departure from the company. Once our assays have been optimized it is Multispan's sole responsibility on how these tests are used and to what end purposes. This area is beyond our scope of research and we have no control on what our assays will be used once optimized.

4.0.2. Ethical

The ethical ramifications of our project revolves in collaborating with customers who have had a good track record and paperwork to prove that their drug compounds required for screening are safe and intended for therapeutic medical uses. The internalization assays we are attempting to engineer are simply a tool used for analysis of data to test for the rate of the drug receptor entering into the cell. As a result, it is both the company and our responsibility to ensure that the screening of a particular drug target will not lead to unintended consequences such as the massive manufacturing of deadly toxins. Our obligation as student researchers is to perfect our assays while most of the ethical decisions lies in the CEO of Multispan. We have not been required to sign non-disclosure agreements; theoretically we can legally share our information and data with anyone we please. Ethically, however, we are required to maintain the integrity of our research by only sharing information with our advising and funding parties.

4.0.3. Manufacturability

To ensure integrity our internalization assays must be reproducible with at least 3 individual experiments ran on separate days. In addition, our service has to be robust with time intervals easily adjustable for testing on new drugs. Multispan's proprietary technology and assay procedures build upon data from previous experiments to confirm the validity of our data. We can also go back to literature to ensure we meet the theoretical internalization rates and concentrations with known ligands in order to ensure accuracy. Multispan's proprietary technology and assay procedures build upon data from previous experiments to confirm the validity of our data. We can also go back to literature to ensure we meet the theoretical internalization rates and concentrations with known ligands in order to ensure accuracy.

4.0.4 Political

Experiments are all conducted at company headquarters without the risk of outside intrusion. Results will be shared amongst the two of us through Dropbox and Google documents as well as with our academic and industry advisors. No one else should know or have access to our data and research without implicit consent from Multispan Inc. Errors is to be expected and necessary to report and for conducting proceeding experiments. Without reporting errors, similar ones may appear again in the future. All of our data, whether valid or not, should be shared amongst ourselves and made our advisors aware of its existence. No data should go without being questioned and analyzed.

4.0.5. Safety

The physical risks of our project are minimal. However, we must focus on confidentiality. Each specific ligand/ receptor is unknown to us because they are proprietary compounds from customers of Multispan. They don't know nor are they allowed to know. Physical safety is not exactly a factor in this project but we are dealing with chemicals in a lab setting which may be flammable

and toxic. Therefore, we must observe good laboratory practice. The confidentiality of this project revolves among the two of us working on it and the company itself. It would then be up to the company's dissection who it shares our findings with.

6.0. Summary and Conclusion

Initially our project goal was to develop internalization assays for the entire prostanoid family. Realistically, this was improbable as our team did not have prior biochemistry background before the project. However, we did not imagine that it would be such a difficult task to validate three receptors for agonist response. The difficulty comes in the constraints of the available literature research on the internalization of prostanoid receptors and which compounds we have available at Multispan Inc. In addition, time was a significant limiting factor as we were balancing between classes, projects, job applications, extracurricular activities, and senior design. We were well aware that the tradeoff of running Multispan Inc.'s platform would be time consuming but the tradeoff would be accuracy to the level of individual cells.

In regards to the receptors which did internalize, over 50% internalization was observed for EP4 and EP1 by being activated by PGE_2 and iloprost respectively. EP4 internalized over the time intervals of 30 and 60 minutes with an EC_{50} value of 5.2 nM and 5.6 nM respectively. Because both of the EC_{50} values fall so close to each other, we cannot dismiss either time points. Most importantly, the EC_{50} value falls within the range of other assays, the deviation in MFI is extremely low for each dilution of the drug, and the experiment is performed in triplicates. For EP1, the receptor internalized after 60 minutes after being activated by iloprost. The experiment is validated because it falls within range of the values presented for both IUPHAR and Multispan Inc.'s database for other assays. In addition, the results were reproducible in 3 experiments. In the third experiment, we observed over 40% internalization for the TP receptor after 120 minutes. It was extremely difficult to find literature research on TP internalization and Multispan Inc. did not have any of the known potent agonists. As a result, we took a bit of a risk and chose PGD_2 with the knowledge of its binding affinity value and chose a starting concentration accordingly. We were able to successfully replicate the experiment twice with an extremely small deviation between the MFI for different

concentrations of the drug compound. More importantly, the EC_{50} value falls a little over the converted value listed from IUPHAR for other assays. Because we were unable to repeat this experiment a third time, we cannot completely state that the assay is validated but at the same time we cannot completely dismiss the results.

Besides EP1, EP4 and TP, we also ran EP2 and DP1 assays but we were unsuccessful in our attempt to internalize the receptors. For the most part in the beginning of the project, we struggled on technique so our results for EP2 was not logical at all. The drug we used for EP2 was iloprost because it was actually the protocol initially developed by Dr. Venkhat. We did not attempt to internalize the receptor again until the spring quarter. The results showed very little internalization with iloprost when we ran it again. For DP1, we were initially able to obtain some promising results with over 30% internalization but the results were not repeatable the second time. This may be due to many factors such as unhealthy cells, inconsistent technique, or the drug compound may have been contaminated.

In the future, we hope that the individuals who continue this project obtain the IC_{50} values for EP1 and EP4 receptors. In addition, we would hope that the next group would repeat our TP assay experiment and finish the validation and move on to obtaining IC_{50} values. By obtaining the IC_{50} value, we will then have the complete assay for each receptor to test on libraries of compounds.

Up to this point, we have been able to utilize Multispan's proprietary cell lines to overexpress a few GPCRs. We have activated EP1, EP4, and TP with iloprost, PGE_2, and PGD_2. In turn, we were able to obtain EC_{50} values and specific time intervals for receptor internalization. With these assays we are attempting an *in vitro* model of an agonist dose response. These assays monitor receptor activation and internalization. In turn, they become powerful tools for the

discovery of novel therapeutics to test against libraries and to screen from a few thousand to millions of compounds.

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7.0 APPENDICES

7.1 Customer Needs Survey

Interviewer: William Truong

Method: E-mail

Dr. Helena Mancebo, CEO Multispan Inc.

1. Who are Multispan's competitors for the internalization assays?

-No one else is offering internalization assay that we know of.

2. What are Multispan's prices per internalization assay for companies?

-We can't really share pricing information. It's privileged information between Multispan and its customers

3. Is time a factor that is weighed heavily for the internalization assays?

It's all driven by biology---i.e. How long it takes for measurable amount of each receptor to be internalized following receptor activation.

4. During this internship at Multispan, what will be required for Josergio and I?

You will need to perform the following:

1. Learn and perfect cell culture, FACS, and assay development techniques.
2. Independently plan and carry out experiments from start to finish.
3. After each experiment, critically analyze data, troubleshoot and design the follow-up experiments.
4. Perform literature search, deepening understanding in receptor protein turnover and trafficking. Seek advice regularly by asking prepared questions.
6. Apply rigor and diligence to your work at all levels.

5. What are the requirements for the results of each internalization assay (customer's perspective)?

1. Reproducibility: Each assay need to be shown with less than 3 fold differences EC50s or IC50s in 3 independent experiments to be considered "validated".
2. Timeline: Develop validated internalization assays for all targets within each assigned receptor family by the end of the project.

Interviewer: William Truong
Method: In-Person
Dr. Ricai Han, Senior Scientist Multispan Inc.

1. Is Multispan aware of any specific information regarding the drugs sent by the companies such as Johnson and Johnson?

Multispan does not know the affinity of the receptor or other specific information about it. We also don't know whether compound could affect trigger signal transduction.

2. Without knowing any specifics about the drugs, what does a researcher need to do in order to perform these internalization assays?

We need to characterize the function and activities of how it would affect surface expression. In addition, we need to find the internalization factor.

3. What is expected of us after we are able to report an internalization assay for one GPCR receptor?

After completing this task we may move onto other assays like cAMP, Beta etc.

4. As a scientist, what is your take on weighing in time as an important variable for these experiments?

It is desired but probably not yet achievable for speeding up the time of internalization for each receptor. Each internalization time varies based on the receptor type.

Interviewer: William Truong, Josergio Zaragoza
Method: In-Person
Dr. Radhika Venkhat, R & D Manager, Multispan Inc.

1. Can you share with us your experience with internalizing receptors?
-I have worked with many GPCRs for internalization but for your assigned family, I have only worked with DP2.

2. What issues did you face during your internalization assays?
The first issue is framing the time point of internalization for an unknown compound with a receptor. The reason is that there is a rapid recycling time frame for some receptors and it is essential to grasp this specific time interval in order for us to not miss the point of internalization.

3. How do you determine the agonists and antagonists that are used for each assay?
Agonists and antagonists are chosen base on those reported from literature research and analysis. Some internalization is derived from external factors. Others may require internalization with other receptors (Ex: some articles have noted that EP2 internalizes with EP4.

4. What signaling pathways did you utilize during your internalization assays?
I only checked on surface independent pathway not overexpressing receptors. I did not check the dependent downstream signaling pathway.

5. What is your advice in your approach towards coming up with an internalization assay for our families of receptors?
Keep practicing on the technique and running the assays. Once you master the technique and read more on the literature you would then learn whether there's a need

to increase or decrease drug concentration and whether the time intervals need to be shorter or longer.

7.1.1 Detailed Product Design Specification

Table A-1. Detailed product design specification breaking down three main categories: assay, confidentiality and deliverables.

Category	Requirement	Importance
Assay	EC50,IC50 must be found to be accurate within a threefold difference	**High-** The minimum threshold of internalization of the receptor with any given agonist must be accurately determined for the assay to be valid.
	Validated internalization assay for all targets in prostanoid Family	**Med-Low-** Some receptors in our family may require external factors or co-internalization with another receptor in order to internalize which is out of our abilities. Not all receptors in all families have been identified, studied, or found.
	Assay must be reproducible in 3 individual runs	**High-** In order to cut down on time, costs, and to have complete validation this must be true
Confidentiality	Proprietary procedures, materials and information must remain within Multispan Inc.	**High-** All recombinant cells and reagents must remain inside the company. Procedures should not be shared with individuals outside of advisors.
	Ligand affinity and properties provided by other companies will not be known and should not be investigated for	**Low-** It is not very likely that we will be working with outside ligands. The properties of these ligands will not be provided.

	purposes outside assays.	
Deliverables	Data must be complete and under final conditions for two receptor families	**High-** Any discrepancies between the data will cause doubt in the user and there needs to be controlled and monitored conditions

7.1.2. Detailed Budget Breakdown

Table A-2. Detailed budget breakdown and costs of the materials, including the vendor, quantity, unit, and description of the item used for research.

Vendor	Description	Price	Unit	Quantity	Cost
VWR	15 mL conical tubes	68.00	500/case	1	$79.75
VWR	96-Well Plates, Polypropylene, V-Bottom	106.64	100/case	1	$144.51
BD Biosciences	Calibrite 3 color beads	257.50	1 box	1	$318.09
VWR	Nonsterile reservoirs	46.64	100/case	1	$78.90
VWR	Hyclone FBS	169.84	1 bottle	3	$541.78
VWR	2 mL pipets	50.38	500/case	1	$59.99
VWR	10 mL pipets	27.85	200/case	1	$37.46
VWR	Trypsin	33.00	6x100mL/pack	1	$52.59
Invitrogen	Gibco DMEM w/ GlutaMAX	196.78	1 case (10x500mL)	1	$230.87
VWR	10 mL pipets	27.85	200/case	1	$41.93
VWR	Dialyzed FBS (hyclone)	385.39	500 mL	3	$1,234.93
VWR	Cryovials	100.35	500/case	1	$179.11
VWR	Eppendorf* Safe-Lock Polypropylene, 0.5 mL	40.80	500/case	1	$47.31
VWR	P1000 tips, with filter, sterile	44.00	768/pack	1	$66.25
VWR	T-25 flasks	67.85	100/case	1	$90.10
VWR	25 mL pipets	68.00	200/case	1	$90.25
VWR	6-well plates	57.50	50/case	1	$79.75

VWR	96-well plates	67.00	50/case	1	$89.25
VWR	15 mL conical tubes	68.00	500/case	1	$90.25
Office Depot	Bleach	2.96		1	$3.48
VWR	DMEM	35.94	6/pack	6	$223.09
VWR	PBS 1X W/O CA+MG 6X500ML	35.94	6x500 mL	1	$43.39
VWR	5 mL pipets	26.60	200/case	1	$44.48
VWR	Sterile reservoirs	95.39	100/case	1	$106.74
VWR	T-75 flasks	63.20	60/case	1	$77.70
VWR	medium Nitrile gloves	132.79	1000/case	1	$144.14
VWR	Universal Fit Tips, Low Retention (p20)	104.00	4800/case	1	$117.53
VWR	96-Well, U-Bottom Assay Plate	125.47	50/case	1	$139.30
VWR	DME/F12 media	8.2	500 mL/each	6	$59.38
VWR	Aspirating pipets	30.20	200/case	1	$40.38
VWR	P20, P200 refills	18.60	960/pack	1	$28.78
VWR	P1000 tips, no filter, non-sterile	10.00	576 tips/pack	1	$20.18
VWR	Conical-Bottom Centrifuge Tube with Flat Cap, 50 mL	80.34	500/case	1	$88.37
ProZyme	anti-FLAG-RPE Conjugate (FACS Ab)	650.00	1 mg/vial	2	$1,403.00
BD	BD FACSFlow Sheath Fluid	31.00	20 L/each	3	$93.00
	Total Expenses				$6,185.9

7.2 Glossary of Terms

Ligand: substance that binds to a chemical entity to form a larger complex

Agonist: chemical that binds to a receptor and triggers a response

Antagonist: ligand that blocks or dampens agonist-mediated responses

EC50: dosage (concentration) which produces 50% of a maximum given effect

IC50: half maximal inhibitory concentration of a compound to inhibit a biological or biochemical effect

Internalization: endocytosis (when cell engulfs molecule or receptor) of GPCR

Clathrin: protein that plays a major role in the formation of vesicles

Arrestin: small family of proteins important for regulating signal transduction

Flow Cytometry: technique for counting and examining microscopic particles by suspending them in a stream of fluid and passing them through an electronic detection zone

7.3. Project Management Timeline

Fall Quarter

Week 5:

- Continue training on cell culturing and shadow Dr. Venkat on cell assay techniques
- Write up budget proposal to submit to both senior design and engineering department for grant
- Continue looking up literature research for prostanoid receptors
- Learn how to use the live cell flow cytometer
- Run first GPCR internalization assay Friday one of our receptors of interest
- Send e-mails or meet up with Researchers for customer needs report

Week 6:

- Review Internalization Assay procedure and notes
- Continue Literature research on the internalization procedures done on two interested families of receptors (EP1 and EP2)
- Work on Customer needs report to submit Friday
- Practice using FACS machine
- Learn how to use FACS software

Week 7:

- Meet up with Dr. Mancebo and Dr. Zhang to talk about our progress and clear up direction for project
- Gather literature research and come up with protocol for internalization assay
- Go over internalization protocol with Dr. Venkat and work on skills required for assay
- Start working on CDR draft

Week 8:

- Continue literature research on EP1 and EP2 and decide whether these two receptors are viable starting point.
- Continue practice with FACS machine, internalization assays and begin looking into protocols for cAMP assays if needed

- Finish CDR draft and turn in Friday

Week 9:

- At this point we should be really familiar with cell culture , passaging of the cells, and become more comfortable with working on internalization assays
- Come up with an alternative method such as modifying agonist or antagonist receptors if internalization assay was not successful for the two target GPCRs up to this point.
- Decide whether or not to move on to another receptor family and receive feedback from our two advisors
- Start practicing for presentation

Week 10:

- Trouble shooting/catch up week
- Continue literature research and move on to another prostanoid receptor to prepare for next quarter
- Presentation slide package due
- Wrap up experiment and submit results and work throughout quarter to adviser to see what direction we need to head to next quarter
- Work on CDR draft to submit

Week 11: (Finals Week)

- Follow up on any work that needs to be completed
- Begin scheduling for working times for next quarter
- Review over data to see if there is a need to rerun any experiment
- Perform research on prostanoid receptor pathway of choice and start on a protocol for Andy and Dr. Mancebo to review before heading off to break

Winter Quarter:

-Start on internalization assays on different receptor of interest

-Work on detailed drawings due near middle of quarter

-Work on assembly drawings and initial hardware due end of quarter

-Work on presentations for senior design

-Work on Poster for engineering events if we are funded by engineering

-Start developing internalization assays on different receptors of interest

-Work on and finish end of quarter report

-Plan schedule for spring quarter and set up new meeting times for working at Multispan and meetings with advisor

Spring Quarter:

-Work on the finalization stages of our assay development

-Continue practicing on public speaking skills for presentation

-Finalize data and results and work on additional receptors if ample time is left and enough literature research is obtained to support assay analysis

-Prepare for senior design conference

-Start early on putting together senior design thesis

-Prepare poster board for open house

-Finalize slides for senior design presentation

-Reserve the last day before the presentation to relax and be ready for the presentation

7.4. Extra Experiments

7.4.1. EP1

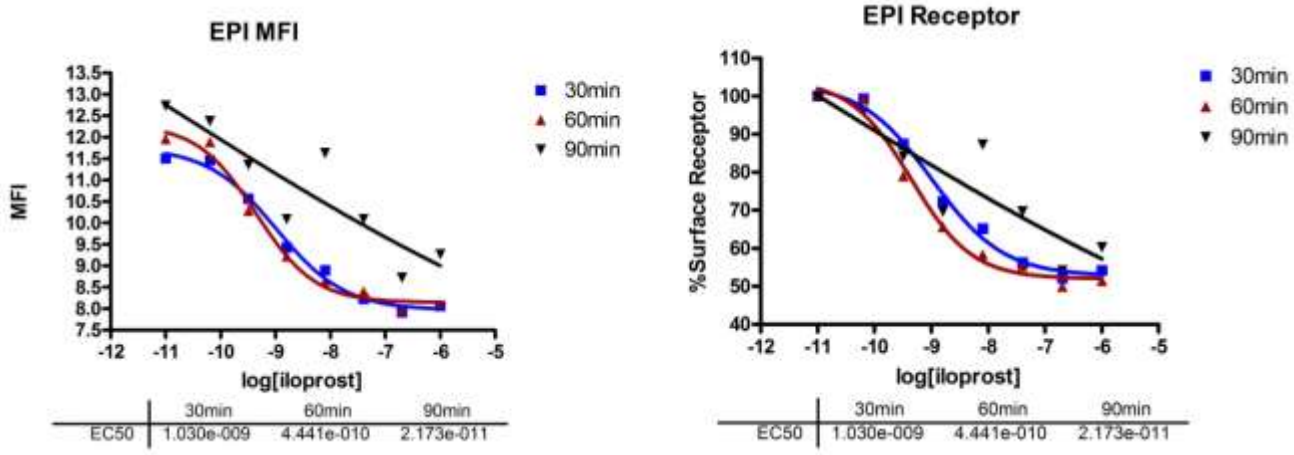

Figure A. Preliminary EC_{50} plot for EP1 which includes 30, 60, and 90 minutes.

7.4.2. EP4

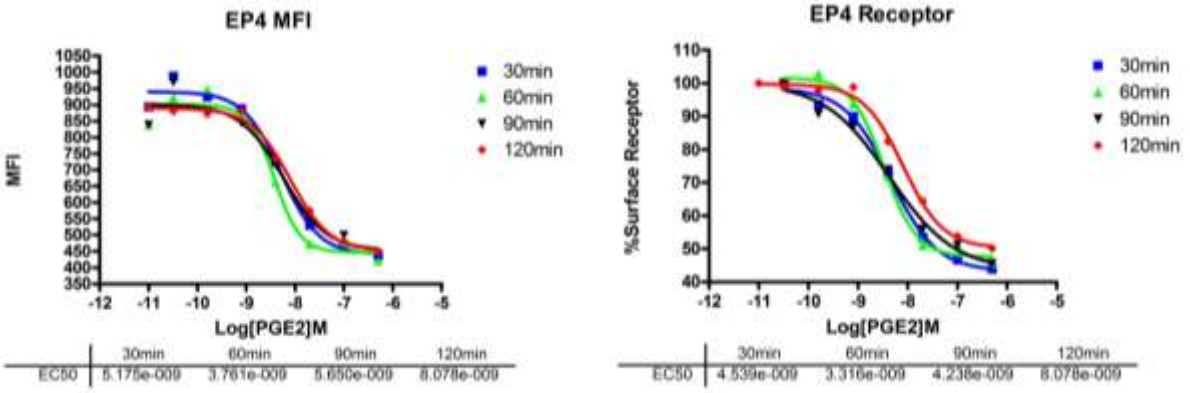

Figure B. Preliminary EC_{50} plot for EP4 which includes 30, 60, 90, and 120 minutes.

7.4.3. DP1

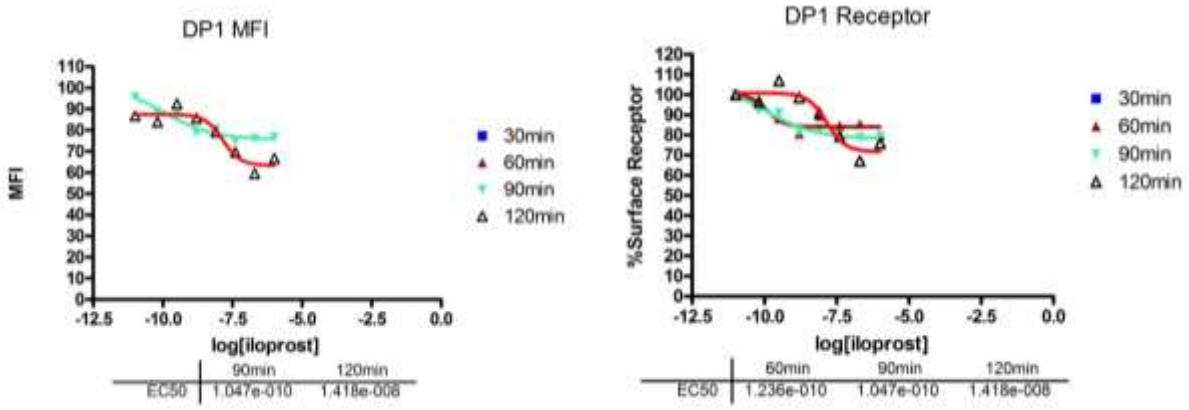

Figure C. DP1 EC_{50} plot which shows only 90 min and 120 minute. Dose response curve cannot be fitted for 30 and 60 minutes.